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A Human Lectin Microarray for Sperm Surface Glycosylation Analysis*[S]

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Glycosylation is one of the most abundant and functionally important protein post-translational modifications. As such, technology for efficient glycosylation analysis is in high demand. Lectin microarrays are a powerful tool for such investigations and have been successfully applied for a variety of glycobiological studies. However, most of the current lectin microarrays are primarily constructed from plant lectins, which are not well suited for studies of human glycosylation because of the extreme complexity of human glycans. Herein, we constructed a human lectin microarray with 60 human lectin and lectin-like proteins. All of the lectins and lectin-like proteins were purified from yeast, and most showed binding to human glycans. To demonstrate the applicability of the human lectin microarray, human sperm were probed on the microarray and strong bindings were observed for several lectins, including galectin-1, 7, 8, GalNAc-T6, and ERGIC-53 (LMAN1). These bindings were validated by flow cytometry and fluorescence immunostaining. Further, mass spectrometry analysis showed that galectin-1 binds several membrane-associated proteins including heat shock protein 90. Finally, functional assays showed that binding of galectin-8 could significantly enhance the acrosome reaction within human sperms. To our knowledge, this is the first construction of a human lectin microarray, and we anticipate it will find wide use for a range of human or mammalian studies, alone or in combination with plant lectin microarrays. *Molecular & Cellular Proteomics 15: 10.1074/mcp.M116.059311, 2839–2851, 2016.*

Glycosylation is one of the most abundant forms of protein posttranslational modifications. It is estimated that more than half of the human proteins are glycosylated with different glycan chains (1). Glycosylation plays critical roles in many biological processes, such as protein folding and conformational stability, cell growth, division, differentiation, and as receptors to sense extracellular signals from surrounding cells or invaded pathogens, which then triggers cellular or immunological responses (2). In addition, the mature coating of glycans on the surface of sperms is a prerequisite for them to gain fertilizing capability (3).

Numerous studies have shown that aberrant glycosylation is highly associated with a variety of diseases, from cancer to neuronal diseases to infertility (3–5). For example, the increase in sialylation in cancers has been shown to promote cancer metastasis and is a strong indicator of poor prognosis (6).

Vertebrates, especially human, have a unique glycan repertoire which is structurally distinct from that of invertebrates (7). Knowledge of the details of the glycan composition, the structures, and the proteins to which they attach will help us understand their function. The identified disease-specific glycosylation may also facilitate the development of accurate diagnosis or effective treatment. However, traditional technologies for glycan analysis, such as mass spectrometry, Western blotting, and chromatography, are time-consuming, costly or require sophisticated handling. Moreover, they are not suitable for rapid and systematic analysis of protein glycosylation (8).

Lectin microarrays were first introduced in 2005 (8, 9). Subsequently, a variety of lectin microarrays have been constructed using plant lectins. These microarrays have been widely applied for the analysis of bacterial glycosylation (10),

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host-pathogen interactions (11), sperm surface glycans (12), cancer biomarker identification (13, 14), metastasis (15), and cancer stem cell analysis (16). Plant lectins can recognize many glycan structures, however, they may not bind with complex glycan structures in mammals, especially humans (17).

There are also many human proteins that bind to glycans: not including glycotransferases, these are considered human lectins or human lectin-like proteins (18). Besides soluble proteins and secreted proteins, many human lectins are membrane proteins (19). There are 4 types of human lectins: C-type, P-type, S-type, and I-type (20). The C-type lectins require calcium for binding and they recognize various sugar chains. P-type lectins are Mannose 6-phosphate receptors, whereas I-type lectins bind with sialic acid and S-type lectins, as galectins, are a family of proteins that bind β-galactoside sugars.

The glycan structures on human glycoproteins are usually more complicated than that of other species. As endogenous proteins, human lectins have higher specificity and stronger adhesive strength with endogenous glycoproteins of human or human pathogens. For example, galectins, as galactose-binding proteins, do not bind epimers of galactose (namely, mannose (21)). Compared with plant lectins, human lectins may provide a better possibility for understanding glycosylation related humeral physiology and pathology.

Sperm is a special type of cell in mammal, which is covered with a layer of glycans, namely, glycocalyx(22). The increase in the abundance of sialic acid on sperm surface is a main indication of sperm maturation (12). For example, glycoprotein CD59 on the surface of acrosome-reacted spermatoza protects the male gametes from complement-mediated damage and gamete adhesion when they travel through the female genital tract (22). The sperm glycosylation also plays key roles for sperm-oocyte binding (22).

To facilitate studies of human glycosylation and to enhance the power of lectin microarrays, herein, we first expressed and purified 60 human lectins and human lectin-like proteins. We then constructed a human lectin microarray with all of these proteins. Taking human sperm as an example, we show that the microarray is capable of revealing functionally important human lectin-sperm surface glycan/glycoprotein interactions. This human lectin microarray is a general platform, which could also be widely applied for other human glycosylation related studies including cancers.

EXPERIMENTAL PROCEDURES

Human Lectin and Lectin-like Proteins—The Gateway entry clones of human lectin and lectin-like proteins were gifts from Dr. Heng Zhu's lab at Johns Hopkins University (Table I). All these clones were sequence verified and subjected for Gateway LR reaction to generate the expression clones using pEGH-A (23) as the destination vector. The expression clones were then transformed into *Saccharomyces cerevisiae* strain Y258 (24).

Fabrication of Human Lectin Microarray—Proteins were expressed and purified as previously described (25). Briefly, all these proteins were expressed with N-terminal glutathione S-transferase (GST) tags and then affinity purified with glutathione agarose beads (26). The purity and concentration of these proteins were estimated by silver staining. The proteins were printed on Polymer-Slide H or Polymer-Slide G (CapitalBio Inc., Beijing, China) along with controls (elution buffer, printing buffer, GST, BSA, and ConA) for 12 identical blocks. Each protein was spotted triplicate in each block using a Smart-Arrayer 48 microarrayer (CapitalBio, Beijing, China). After immobilization overnight at 4 °C, the microarrays were then stored at −80 °C prior to use.

Incubation of Cell Lysate with Human Lectin Microarray—THP-1 cells (2×10^6) were harvested and treated with Nonidet P-40 lysis buffer (Tiangen Biotech, Beijing, China). Two aliquots of the lysate were prepared. One aliquot was subjected for labeling with DyLight 550 NHS Ester (Thermo Scientific, Bremen, Germany) at a ratio of 1:100 (v/v), and the other aliquot was treated with 6.25 U/μl PNGase F (New England BioLabs, Ipswich, UK) for 2 h at 37 °C and then labeled with Dylight 550 following exact the same procedure. Zeba™ Desalt Spin Columns (Thermo Scientific, Bremen, Germany) were used to remove excess free- DyLight 550 NHS Ester. The labeled cell lysates were incubated with the human lectin microarray for 2 h at room temperature, and then washed three times with PBST. The microarrays were air-dried and scanned with a GenePix 4200A microarray scanner (Molecular Devices, Abingdon, UK). Data were processed with GenePix Pro 6.0 (Molecular Devices, Abingdon, UK). The signal intensity of each lectin was averaged from the three replicate spots. Signal-to-noise ratio (SNR) was defined as signal/standard deviation of background, and it was set as the final signal. The cutoff was defined as SNR $\geq$4.

Sperm Collection and Preparation—Human semen samples were collected from Shanghai Jiai Genetics & IVF Institute. According to the fifth edition of WHO laboratory manual for the examination and processing of human semen, the semen samples were evaluated for volume, sperm density, percentage of motile sperm after liquefying for 30 min. Selecting the Sperm which had normal sperm count (sperm count $\geq 1.5 \times 10^7$/ml) and motility (progressive motility (PR $\geq$ 40%)) for this research. The sperm cells were collected by centrifugation (500 g, 10 min) from the semen, after washed 3 times with PBS, collected sperm was fixed with 2% paraformaldehyde/0.2% glutaraldehyde for 30 min, and then stored at 4 °C in order to the subsequent experiments (12).

Incubation of Cells with Human Lectin Microarray—The fabrication of the lectin microarray and profiling of live cells was performed as previously described (12, 16). Briefly, the cells were harvested and fluorescently labeled with CellTracker™ Orange CMRA (Thermo Scientific, Bremen, Germany). Fixed sperm were labeled with 20 μg/ml propidine iodide (PI) for 20 min at room temperature. The cells were then re-suspended in binding buffer (PBS with 0.5 mM $CaCl_2$, 0.1 mM $MnCl_2$ and 1% BSA.) and 1×10^6 cells were probed per block on the human lectin microarray. After incubation for 1 h at room temperature, the microarray was washed in PBST. This microarray was scanned with a GenePix 4200A scanner and the data was processed with GenePix Pro 6.0. The data analysis was similar as previously mentioned, at the same time each positive lectin spot should be observed under microscopy.

Flow Cytometry Analysis and Immunofluorescence of Human Lectin-Sperm Binding—Human sperms of 5×10^6 were re-suspended in PBS and incubated with biotinylated human lectin at a concentration of 1 μM for 2 h at room temperature. The sperm samples were washed 3 times with PBS and resuspended in 100 μl PBS. Cy3-steptavidin was added to the supernatant at a ratio of 1:100 (v/v) for 1 h, and then labeled with DAPI at room temperature for 0.5 h. After washed with

PBS, the sperm samples were re-suspended with ddH_2O and subjected for analysis by a confocal laser scanning microscopy A1Si (Nikon, Tokyo, JP). Meanwhile, the sperm samples were also analyzed with a FacsCalibur™ Flow cytometer using CellQuest software from BD (Franklin Lakes, NJ).

Capture of Sperm Glycoproteins by Human Lectin Affinity—Liquefied semen was centrifuged and the seminal plasma was discarded followed by 3 washes with PBS. 1×10^8 sperm samples were lysed by RIPA(Beyotime Biotechnology, Jiangsu, CN) according the manufacturer's menu. Glutathione agarose beads were coated with human lectin and GST protein. Sperm lysate was equally separated into two aliquots, one aliquot was incubated with agarose beads coated with human lectin at a concentration of 1 mg/ml. The second aliquot was incubated with glutathione agarose beads coated with GST only as control. The beads were washed three times with lysis buffer, and the samples were then subjected for silver staining.

LC-MS/MS and Data Analysis—For LC-MS/MS, the assay was performed as described with slight modifications (15, 27). In brief, the sample was separated by 10% SDS-PAGE gel and was cut each band and then diced into 1.5 ml tubes. Approximately 1 mm cubes were destained by incubation in 100 μl destaining solution, which is made with the same volume of 50 mM sodium thiosulfate and 15 mM potassium ferricyanide. After destained, the gel pieces were reduced by incubation in a solution of 10 mM DTT in 25 mM ammonium bicarbonate at 60 °C for 60 min. For alkylation of proteins, the gel was incubated in a solution of 25 mM iodoacetamide at room temperature for 20 min, followed by washing using 50 mM ammonium bicarbonate and 100% acetonitrile respectively. After dehydration in 100% acetonitrile for 5 min, gel pieces were completely dried by SpeedVac. Then the gel pieces were swollen in 30 μl of 25 mM ammonium bicarbonate buffer containing 0.01 mg/ml Sequencing Grade Modified Trypsin (Promega, Madison, WI) and incubated overnight at 37 °C. Peptides were extracted with 60% acetonitrile containing 5% formic acid two times and 100% acetonitrile one times, dried by vacuum centrifugation at 50 °C, and stored at −20 °C for further analysis. The peptide samples were analyzed using an ESI-Q-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) combined with an UltimateTM 3000 nano-LC system (Dionex, Sunnyvale, CA). Each peptide sample was redissolved in 2% acetonitrile with 0.1% formic acid, and then loaded onto a Peptide Captrap column (Michrom Bioresources Inc., Auburn, CA) with the autosampler of the MDLC system (Michrom Bioresources Inc.). Then trapped peptides were released and separated on a C18 capillary column (0.1 mm i.d.×150 mm, 3 μm, 200 Å, Michrom Bioresources Inc.). The peptides were separated using a solvent system with solvent A consisting of 99.9% water and 0.1% formic acid, and solvent B consisting of 99.9% acetonitrile and 0.1% formic acid. The peptides were eluted with gradient 2–10%B in 6 min, 10–35%B in 34 min, 35~90% in 5 min and 90% hold for 2 min with a constant flow rate of 500 nL/min. The LC setup was coupled online to a Q-TOF using a nano-ESI source (Bruker Daltonik) in data dependent acquisition mode (*m/z* 350–1500). The Source Capillary was set at 2000 V, the flow and temperature of dry gas was 2.0 L/min and 150 °C respectively. The mass spectrometer was set as one full MS scan followed by ten MS/MS scans on the ten most intense ions from the MS spectrum with the following dynamic exclusion settings: repeat count = 2, repeat duration = 15 s, exclusion duration = 30 s.

The raw data were extracted using LC/MS software DataAnalysis 4.1 (Bruker Compass software) and converted into mgf files for further MASCOT 2.4 (Matrix Science, Boston, MA) search. Mascot was set up to search the SwissProt 2014_04 *Homo sapiens* database (20266 entries). Carbamidomethyl of cysteine was specified as fixed modifications, and oxidation of methionine and N-terminal acetylation were specified as variable modifications. Trypsin specificity was used, allowing for two missed cleavages, and a mass tolerance of 20 ppm was used for MS precursors and 0.05 Da for fragment ions. Peptide charges of + 2, + 3, and + 4 were selected.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (28) partner repository with the data set identifiers PXD003785 and 10.6019/PXD003785.

Experimental Design and Statistical Rationale—A total of five Galectin-1 specifically enriched bands that represented a wide range of molecular weight were subjected for mass spectrometry identification. After digestion, the digested product of each band was analyzed once by mass spectrometry. The threshold score was greater than or equal to 30, expectation value was 0.05 and the number of peptides was more than 2 for accepting individual spectra.

In Vitro Sperm Capacitation and Acrosome Reaction Assay—For *in vitro* acrosome reaction (AR)[1] assays, human semen samples were processed by gradient separation using ISolate (Irvine Scientific, Santa Ana, CA). Dead sperm and debris were found in the upper portion of the tube whereas a fraction of highly motile, morphologically normal sperms formed a pellet in the bottom. Sperms of 5×10^6 were recovered in 0.1 ml of human tubal fluid (HTF) capacitation medium with 5% human serum albumin (HSA) and 1 μM galectin-8 or Glutathione S-transferase was added to the medium. Sperm suspensions were then incubated for 3 h at 37 °C in an atmosphere of 5% CO_2 in air. For induction of AR, 40 mM progesterone (P4) (Sigma-Aldrich, Saint Louis, MI) were added, and sperm were incubated 30 min at 37 °C and 5% CO_2. Peanut agglutinin (PNA) was used to assess the sperm acrosomal status as described elsewhere (29).

RESULTS

Fabrication of the Human Lectin Microarray—The overall workflow of the construction and application of the human lectin microarray is shown in Fig. 1. Briefly, after the human lectins or lectin-like proteins were expressed and purified, the proteins were printed onto appropriate substrate slides for fabrication of the lectin microarray. The lectin microarray could then be subjected to a variety of applications, including sperm analysis.

To construct the microarray, we first set out to collect as many clones of human lectin or lectin-like protein as possible. 93 clones were obtained from several sources, including a few clones that were constructed by ourselves. All of these clones were Gateway entry clones. pEGH-A (23) with a N-terminal GST fusion was chosen as the destination vector for constructing the expression clones by the Gateway LR reaction. After several rounds of attempts, we successfully purified 60 human lectins and lectin-like proteins in a soluble form by GST affinity purification (Table I). Silver staining showed that most of the proteins are of the expected size, and their purity is suitable for microarray construction (Fig. 2*A*).

It is a common practice to test a set of surface chemistries when fabricating a new type of protein microarray or a new application. Several types of substrate slides were thus examined for optimal functionality in the human lectin microarray. Based on the spot morphology and the immobilization

[1] The abbreviations used are: AR, Acrosome reaction; AI, Acrosomal intactness; HSP90, Heat shock protein 90; PI, propidine iodide; PNA, Peanut agglutinin.

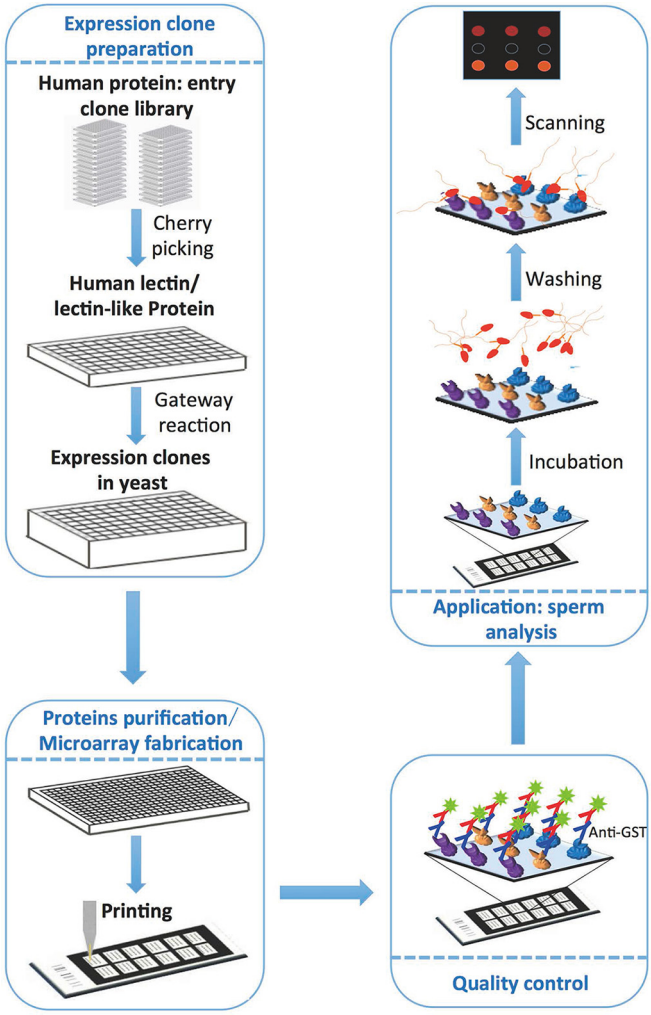


FIG. 1. **The workflow of the construction and application of human lectin microarray.**

efficiency of the human lectins (data not shown), a substrate slide coated with the polymer, Polymer-Slide H from Capitalbio, was chosen for this study. All the 60 purified proteins were printed on the microarray along with controls. To assure the reproducibility and to reduce the cost, 12 identical blocks were printed on a single microarray, and each protein was printed in triplicate.

To check the overall quality, the human lectin microarray was probed with an anti-GST antibody followed by incubation with a fluorescently labeled secondary antibody. The results showed that the printing and immobilization were reproducible among all of the 12 blocks on a single microarray. Also, substantial immobilization of human lectins and lectin-like proteins was obtained (Fig. 2*B*). Although the signal intensity varied among the lectins, the signal intensities for most of the lectins were significantly higher than that of the background (Fig. 2*C*), including 36 membrane proteins that are traditionally challenging to be overexpressed and purified.

The Majority of Lectins on the Microarray are Active—It is a general practice to confirm the activities of proteins on the protein microarray once a new type of microarray is constructed. Additionally, among the final set of 60 successfully purified human lectins and lectin-like proteins, 36 of them localize to the membrane. It is well recognized that membrane proteins are not stable, especially after purification. Thus, it is necessary to confirm the overall activities of the lectins on the human lectin microarray to assure its applicability. However, because of the extreme complexity of the human glycan structures and composition, the glycan or binding specificities of most of the human lectins and lectin-like proteins are not well annotated. Thus it is not feasible to confirm their glycan binding activities by testing appropriate corresponding glycans one-by-one on the microarray or in a tube.

To confirm the glycan binding activities of the human lectins on the microarray in a more efficient way, a cell lysate that contains a mixture of complex glycans was tested on the microarray. In particular, THP-1 cells were lysed under mild conditions, and after fluorescent labeling, one aliquot of the lysate was probed on the microarray whereas another aliquot that was treated with PNGase F was included as control. Clear bindings were observed on the microarray (Fig. 3*A*). Using a signal to noise ratio (SNR) ≥ 4 as cut off, 26 lectins showed clear binding with the cell lysate, including 10/36 of the lectins that localize to the membrane and 16/24 of the secreted proteins (Fig. 3*B*). As expected, the binding intensities of the majority of the positive binding lectins were reduced significantly upon PNGase F treatment. The most significant signal reductions were from COLEC11v1, LGALS13, and MASP1v3. These results suggested that at least these 26 lectins are active and could specifically bind glycans because PNGase F treatment specifically removes N-glycans from glycosylated proteins.

To further confirm whether the human lectin microarray is also applicable for intact cell analysis similar to the plant lectin microarray (13, 14), two cell lines, 293T and MDA-MB-231, were fluorescently labeled and probed on the microarray as previously described (16). Further, to test the possible application of the human lectin microarray for studies of the host-pathogen interactions, we chose budding yeast as a model and incubated the cells on the microarray following a previously described protocol (30). Obvious but distinct binding patterns were observed among the three types of cells. The binding patterns are more similar between 293T and MDA-MB-231 than that of yeast (supplemental Fig. S1, and supplemental Table S1). Together with the results of the THP-1 cell lysate, there are at least 43 lectins in this microarray that show binding activity.

Human Lectins Specifically Bind Human Sperms on the Microarray—Because of the critical roles that surface glycans play during fertilization, sperm samples from healthy male were chosen for this study and sperm cell have been analyzed by plant lectin microarray(31). To identify the glycans on the

TABLE I
The list of human lectin and lectin-like proteins on the microarray

NO.	Accession	Gene symbol	Definition	Location	Conc. ng/μl	MW(+GST) KDa
1	BC067423.1	B3GNT3	UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 3, mRNA	Golgi apparatus membrane	10	69
2	BC022278.1	CD207	CD207 molecule, langerin, mRNA	Membrane	30	62
3	NM_000450.1	SELE	selectin E (endothelial adhesion molecule 1), mRNA	Membrane	20	141
4	NM_0004066.1	CHODL	Chondrolectin, mRNA	membrane	30	57
5	NM_016511.2	CLEC1A	C-type lectin domain family 1, member A, mRNA	Membrane	400	58
6	BC029554.1	CLEC1B	C-type lectin domain family 1, member B, mRNA	Membrane	200	48
7	NM_005127.2	CLEC2B	C-type lectin domain family 2, member B , mRNA	Membrane	100	43
8	NM_013269.3	CLEC2Dv1	C-type lectin domain family 2, member D transcript variant 1, mRNA	Cell membrane	20	48
9	NM_000655.3	SELL	selectin L (lymphocyte adhesion molecule 1), mRNA	Membrane	60	107
10	BC019883	CLEC2Dv2	C-type lectin domain family 2, member D, mRNA	Cell membrane	60	44
11	NM_003006.3	SELPLG	selectin P ligand, mRNA	Membrane	200	95
12	BC104414.2	CLEC3A	C-type lectin domain family 3, member A, mRNA	Secreted	30	48
13	BC011024.1	CLEC3B	C-type lectin domain family 3, member B , mRNA	Secreted	300	46
14	NM_016184.3	CLEC4A	C-type lectin domain family 4, member A, transcript variant 1, mRNA	Membrane	30	41
15	NM_080387.4	CLEC4D	C-type lectin domain family 4, member D, mRNA	Membrane	50	56
16	NM_014358.2	CLEC4E	C-type lectin domain family 4, member E, mRNA	Membrane	100	61
17	NM_013252.2	CLEC5A	C-type lectin domain family 5, member A, mRNA	plasma membrane	30	47
18	NM_053003.2	SIGLEC12	sialic acid binding Ig-like lectin 12, transcript variant 1, mRNA	Membrane	80	131
19	ENST00000396480	CLECSF12	Dendritic cell-associated C- type lectin 1 (Dectin-1) (Beta-glucan receptor)	Membrane	50	35
20	NM_197954.2	CLEC7Av6	C-type lectin domain family 7, member A, transcript variant 6, mRNA	Membrane	1000	35
21	ENST00000391797	SIGLEC6	Sialic acid-binding Ig-like lectin 6 precursor (Obesity- binding protein 1)	Cell membrane, Secreted	40	82
22	BC013385	CLEC7A	C-type lectin domain family 7, member A	Membrane	40	36
23	NM_207345.2	CLEC9A	C-type lectin domain family 9, member A, mRNA	Membrane	20	53
24	BC103815.1	COLEC10	collectin sub-family member 10 (C-type lectin), mRNA	Secreted	200	57
25	NM_024027.3	COLEC11v1	collectin sub-family member 1, transcript variant 1, mRNA	Secreted	600	56
26	NM_001344.1	DAD1	defender against cell death 1, mRNA	Membrane	1200	40
27	NM_002003.2	FCN1	ficolin (collagen/fibrinogen domain containing) 1 , mRNA	Secreted. Cell membrane	100	62
28	NM_004108.2	FCN2	ficolin (collagen/fibrinogen domain containing lectin) 2 (hucolin), mRNA	Secreted	100	70
29	NM_173452.1	FCN3	ficolin (collagen/fibrinogen domain containing) 3 (Hakata antigen), mRNA	Secreted	60	61
30	BC036390.2	GALNT4	UDP-N-acetyl-α-D-galactosamine: polypeptide N-acetylgalactosaminyltransferase 4, mRNA	Golgi apparatus membrane	10	92
31	BC035822	GALNT6	UDP-N-acetyl-α-D-galactosamine: polypeptide N-acetylgalactosaminyltransferase 6	Golgi apparatus membrane	200	68
32	NM_021808.2	GALNT9vB	UDP-N-acetyl-α-D-galactosamine: polypeptide N-acetylgalactosaminyltransferase 9, mRNA	Golgi apparatus membrane	20	52
33	BC036082.1	HSPC159	galectin-related protein, mRNA	intracellular	1000	59
34	NM_002259.3	KLRC1v1	killer cell lectin-like receptor subfamily C, member 1, transcript variant 1, mRNA	Membrane	30	52
35	NM_007328.2	KLRC1v2	killer cell lectin-like receptor subfamily C, member 1, transcript variant 2, mRNA	Membrane	80	52
36	BC098166.1	KLRF1	killer cell lectin-like receptor subfamily F, member 1, mRNA	Membrane	40	40
37	BC053319.1	SIGLEC8	sialic acid binding Ig-like lectin 8, mRNA	Membrane	400	81
38	BC012621.1	KLRG1	killer cell lectin-like receptor subfamily G, member 1, mRNA	Cell membrane	60	48
39	NM_007360.1	KLRK1	killer cell lectin-like receptor subfamily K, member 1, mRNA	Cell membrane	80	68
40	NM_002543.3	OLR1	oxidized low density lipoprotein (lectin-like) receptor 1, mRNA	Cell membrane, Secreted	20	56
41	NM_005561.3	LAMP1	lysosomal-associated membrane protein 1, mRNA	Cell membrane	20	71
42	NM_013995.1	LAMP2	lysosomal-associated membrane protein 2 , transcript variant LAMP2B, mRNA	Cell membrane	120	75
43	NM_002305.3	LGALS1	lectin, galactoside-binding, soluble, 1 (galectin 1) ,mRNA	Secreted	1000	40
44	NM_033101.2	LGALS12	lectin, galactoside-binding, soluble, 12 (galectin 12), mRNA	Nucleus	40	64
45	NM_013268.2	LGALS13	lectin, galactoside-binding, soluble, 13 (galectin 13), mRNA	soluble	2000	48
46	BC022257.1	LGALS14	lectin, galactoside-binding, soluble, 14, mRNA	Nucleus	1000	42
47	NM_203471.1	LGALS14V2	lectin, galactoside-binding, soluble, 14, transcript variant 2, mRNA	Nucleus	600	45

TABLE I—*continued*

NO.	Accession	Gene symbol	Definition	Location	Conc. ng/μl	MW(+GST) KDa
48	BC059782	LGALS2	lectin, galactoside-binding, soluble, 2(galectin 2) ,mRNA	soluble	1000	45
49	NM_139208.1	MASP2v2	mannan-binding lectin serine peptidase 2, transcript variant 2, mRNA	Secreted	30	102
50	NM_006610.2	MASP2v1	mannan-binding lectin serine peptidase 2, transcript variant 1, mRNA	Secreted	200	47
51	NM_002307.2	LGALS7	lectin, galactoside-binding, soluble, 7 (galectin 7), mRNA	Cytoplasm, Nucleus, Secreted	80	41
52	NM_201543.1	LGALS8	lectin, galactoside-binding, soluble, 8 (galectin 8), mRNA	Cytoplasm	800	61
53	BC016486.2	LGALS8v2/3	lectin, galactoside-binding, soluble, 8 (galectin 8), transcript variant 2/3, mRNA	Cytoplasm	1000	61
54	BC032330.1	LMAN1	lectin, mannose-binding 1, mRNA	membrane	20	100
55	NM_006816.1	LMAN2	lectin, mannose-binding 2, mRNA	membrane	60	66
56	NM_030805.2	LMAN2L	lectin, mannose-binding 2-like, mRNA	membrane	40	79
57	NM_080600.1	MAG	myelin associated glycoprotein , transcript variant 2, mRNA	membrane	200	90
58	NM_001031849.1	MASP1v3	mannan-binding lectin serine peptidase 1, transcript variant 3, mRNA	Secreted	200	68
59	BC106945	MASP1	mannan-binding lectin serine peptidase 1 (C4/C2 activating component of Ra-reactive factor)	Secreted	60	106
60	NM_002580.1	REG3A	regenerating islet-derived 3 alpha, transcript variant 1, mRNA	Secreted	40	46

sperm surface that bind human lectins, the sperms were first fixed and then fluorescent labeled with PI and incubated on the human lectin microarray with 60 lectins and lectin-like proteins. Positive bindings were observed for 5 of the proteins: namely GalNac-T6, galectin-1, ERGIC-53, galectin-8 and galectin-7 (ranked high to low according to their binding intensity on the microarray) (Fig. 4*A*). GalNAc-T6 and ERGIC-53 are membrane proteins whereas the other three are secreted proteins. Because galectins are specific for galactoside and galectin-8 is specific for sialic acid, these results indicate that galactoside and sialic acid on sperm surface may be critical for sperm function.

To verify the lectin microarray results, all five positive bindings on the microarray were evaluated by flow cytometry (FCM) using biotinylated lectins, followed by Cy3-labeled streptavidin for detection. Lectin CLEC-2 that did not show positive binding to sperms was included as a negative control. As shown in Fig. 4*B*, the flow cytometry results are indeed consistent with those of the lectin microarray, with the highest binding observed for galectin-8.

To further validate the microarray results, the sperms were also directly visualized by fluorescent microscopy after incubation with biotinylated lectins followed by Cy3-streptavidin. As shown in Fig. 4*C* and 4*D*, the binding properties from the microarray experiments and those of the flow cytometry analysis were also confirmed by direct inspection of these labeled sperms. Interestingly, different lectins exhibited different binding distributions on the sperm. For example, galectin-8 showed strong binding across the whole sperm, whereas ERGIC-53 bound only the sperm neck and head, with the strongest binding observed on the neck. These results indicate that the glycoproteins that these lectins bind have distinct distributions on the sperm surface.

Galectin-1 Binding Glycoproteins Were Identified by Mass Spectrometry—To identify the glycoproteins on the surface of the sperms that were specifically bound by the human lectins, we focused on galectin-1. Galectin-1 was incubated with the sperm lysate and galectin-1 bound proteins were pulled down through affinity binding between GST tag at the N-terminal of galectin-1 and glutathione beads (Fig. 5*A*). The enriched specific bands were cut from the gel, digested and subjected for LC-MS/MS analysis (Fig. 5*B*). Eight membrane-associated and secreted proteins were successfully identified (Table II, supplemental Fig. S2 and supplemental Table S2) with high stringency. Surprisingly, among these proteins, the heat shock proteins, HSP90 and HSP70, were highly enriched.

To further confirm the identity of these proteins, we focused on HSP90. A highly specific anti-HSP90 antibody was applied (Fig. 5*C*). Sperms were stained with biotinylated galectin-1 and anti-HSP90 simultaneously, and the presence of galcetin-1 and HSP90 were then identified by Cy3-streptavidin and a Cy5 conjugated second antibody that was specific for the anti-HSP90, respectively. According to references and our experience, this fixation treatment will not increase sperm

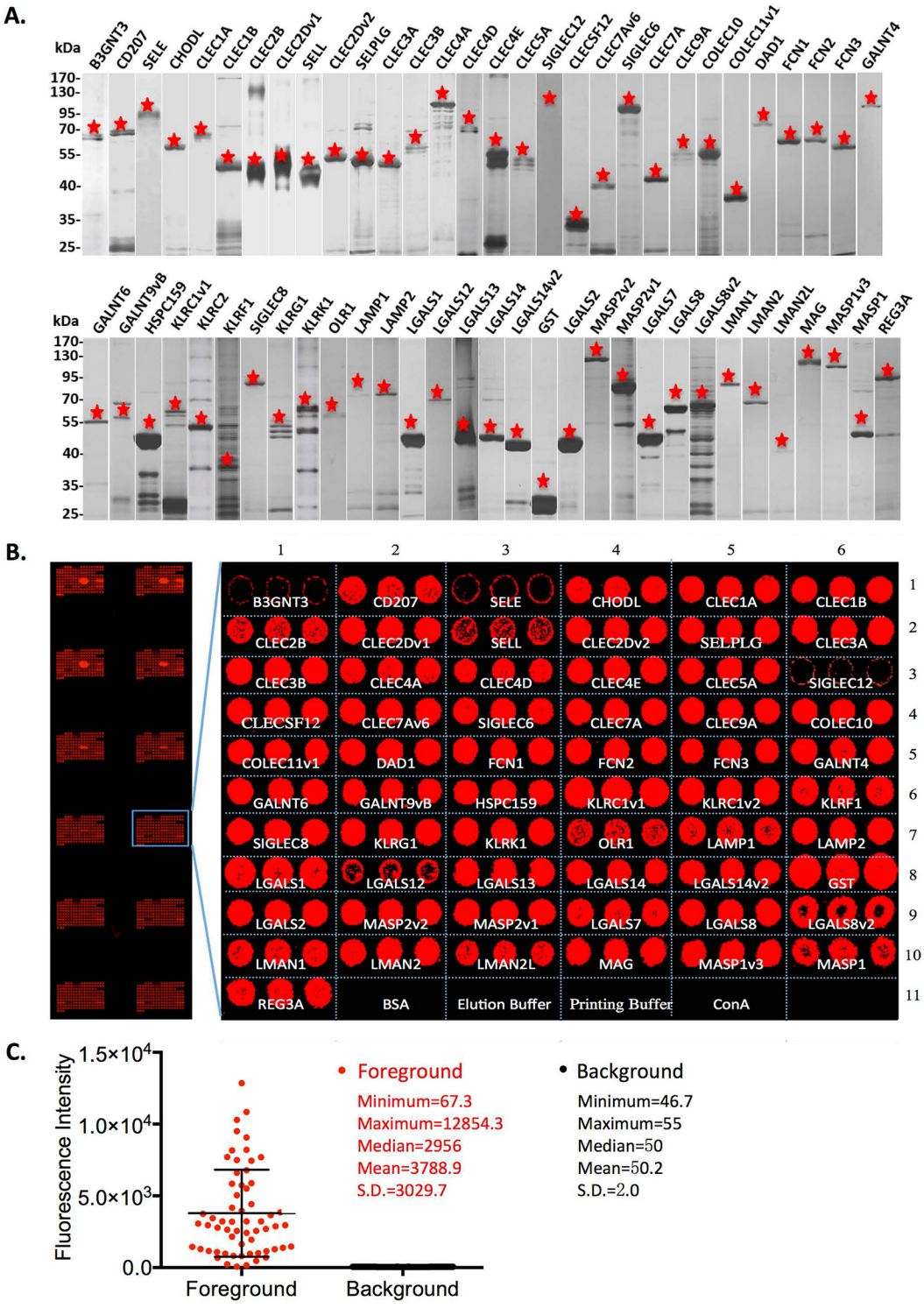


FIG. 2. **The fabrication and quality control of the human lectin microarray.** *A*, Purification of recombinant human lectin or lectin-like proteins. All the proteins were affinity purified from *Saccharomyces cerevisiae* through the N-terminal GST tag. The quality of these proteins was then determined by silver staining using an anti-GST antibody. Stars mark proteins that are of the expected molecular weight. *B*, All the proteins were spotted in triplicate on polymer slides (Polymer- Slide H, CapitalBio). Twelve identical blocks were spotted per slide. To visualize the overall quality of the human lectin microarray, the slides were probed with an anti-GST antibody after immobilization. *C*, Histogram of foreground and background signal intensities shows that most of the printed spots contain substantial levels of recombinant human lectin or lectin-like protein. SNR = Signal intensity/Standard deviation of background intensity.

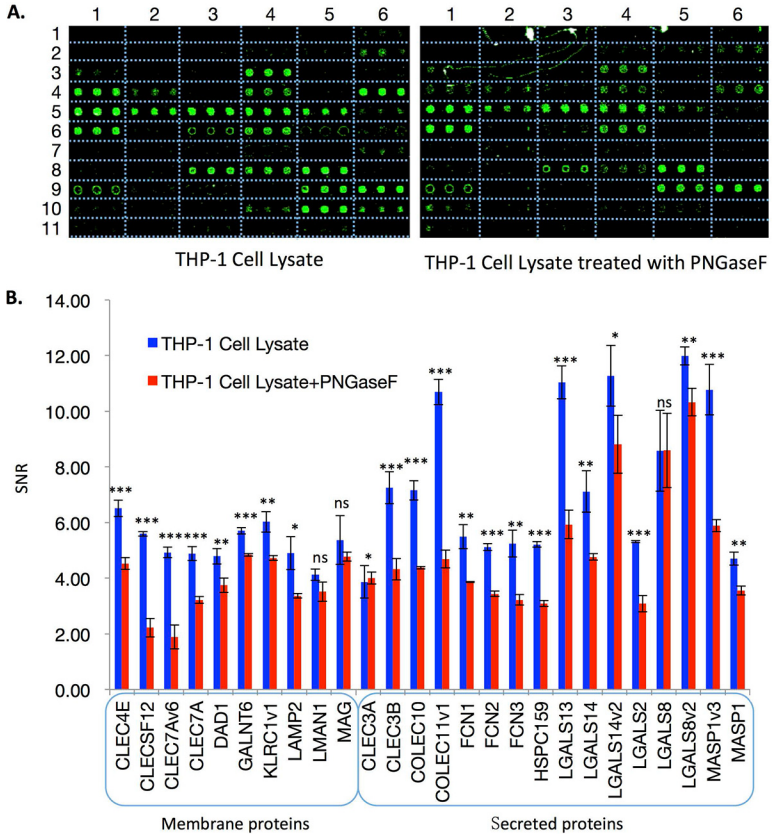


FIG. 3. **Most of the human lectins on the microarray are active.** *A*, Cell lysate (THP-1) treated with or without PNGaseF were probed side by side on the human lectin microarray. The total proteins in the cell lysate were biotinylated prior to microarray incubation. The microarray results were visualized by incubation with a Cy3 conjugated streptavidin. *B*, Quantification of the microarray results. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Error bars represent standard deviations of three repeats.

permeability. The images clearly demonstrated that galectin-1 and HSP90 were co-localized in sperm neck (Fig. 5*D*). These results strongly suggest that HSP90 is a glycoprotein that can be specifically bound by galectin-1 on the sperm. These results are also consistent with the notion that sperm surface chaperones may also possess functions as adhesion molecules that mediate the recognition of sulfoglycolipids during gamete binding (32).

Galectin-8 Enhance Acrosome Reaction of Human Sperm in vitro—The results of lectin microarray and validation indicate the presence of human lectin specific glycans/glycoproteins on the sperm surface. We wondered whether the bindings of the human lectins and the glycans/glycoproteins might contribute to the maturation and function of sperms, such as for example, in the AR, which is a key exocytotic event essential for different stages of the fertilization process. We chose galectin-8, which showed high binding to sperms both on the microarray and in the other validations, to test whether lectin binding affects processes associated with sperm maturation. Two aliquots of equal amounts of sperms were prepared. One aliquot was set for capacitation in the presence of galectin-8, and the other was set for capacitation with the addition of GST as a control. After capacitation, the sperms were induced for the acrosome reaction. The acrosome reaction was then visualized and measured by staining with FITC conjugated PNA (Fig. 6*A*). Two hundred sperms were counted for the sperms incubated with galectin-8 during capacitation or that of the GST control. The sperms of both acrosomal intactness (AI) and AR were counted. The results showed that the addition of galectin-8 during capacitation could significantly promote acrosome reaction (Fig. 6*B* and 6*C*). These results indicate that specific human lectin binding on the sperm surface could have significant functional effects on key biological processes of sperm.

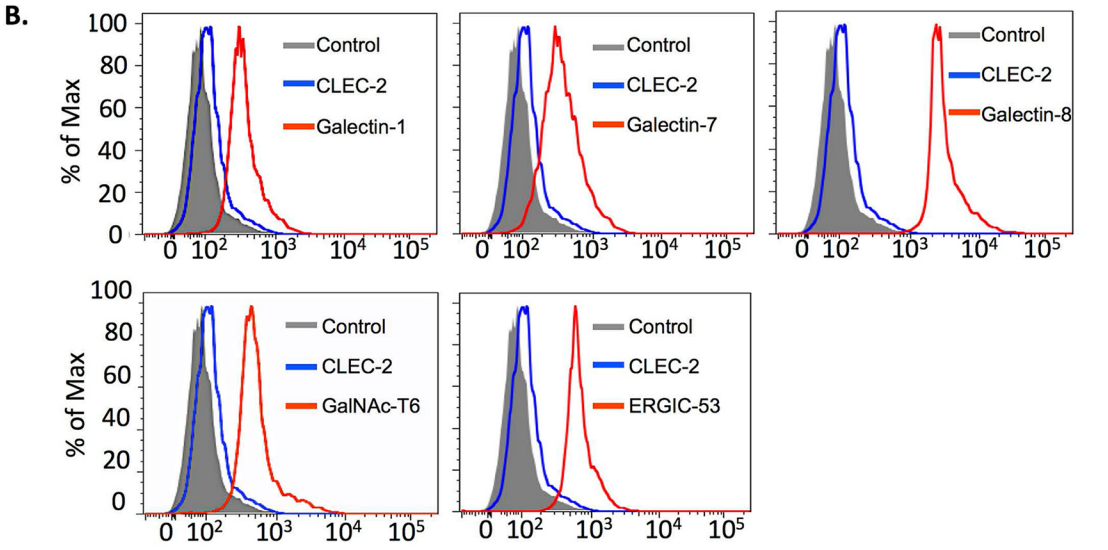
DISCUSSION

Glycosylation plays critical roles in many biological processes. Aberrant glycosylation is closely related to a variety of human diseases. To facilitate the analysis of human glycans, we constructed a human lectin microarray with 60 human lectins and lectin-like proteins. Based on the validations, most of the proteins on the microarray were active. Taking human sperm as an exemplary samples, the lectin microarray experiments determined that GlaNAc-T6, galectin-1, -7, -8 and ERGIC-53 specifically bind sperm and galectin-8 promotes sperm acrosome reaction.

The five positive lectins and lectin-like proteins are specific for three types of glycans, namely sialic acid, galactose, and mannose. Galectins are a family of carbohydrate-binding proteins with an affinity for β-galactosides. Galectin-1 is differentially expressed in various normal and pathological tissues and appears to carry a wide range of biological activities (33), including maternal fetal tolerance, regulation of growth, differentiation, invasion, and immune evasion mechanisms of

A.

Sperm binding	Protein name	Preferred glycan structure	Function	SNR
	GalNAc-T6	GalNAc	Transfer GalNAc to serine and threonine residents	96.02 ± 13.03
	Galectin-1	Galβ4Glc	Modulate cell-cell and cell-matrix interactions	24.40± 1.56
	ERGIC-53	Mannose	Plant lectin homologs in the secretory pathway	11.74 ± 3.30
	Galectin-8	Siaα3Galβ4Glc	Beta-galactoside-binding proteins	67.18 ± 8.20
	Galectin-7	Galβ3GlcNAc	Beta-galactoside-binding proteins	4.77 ± 0.60



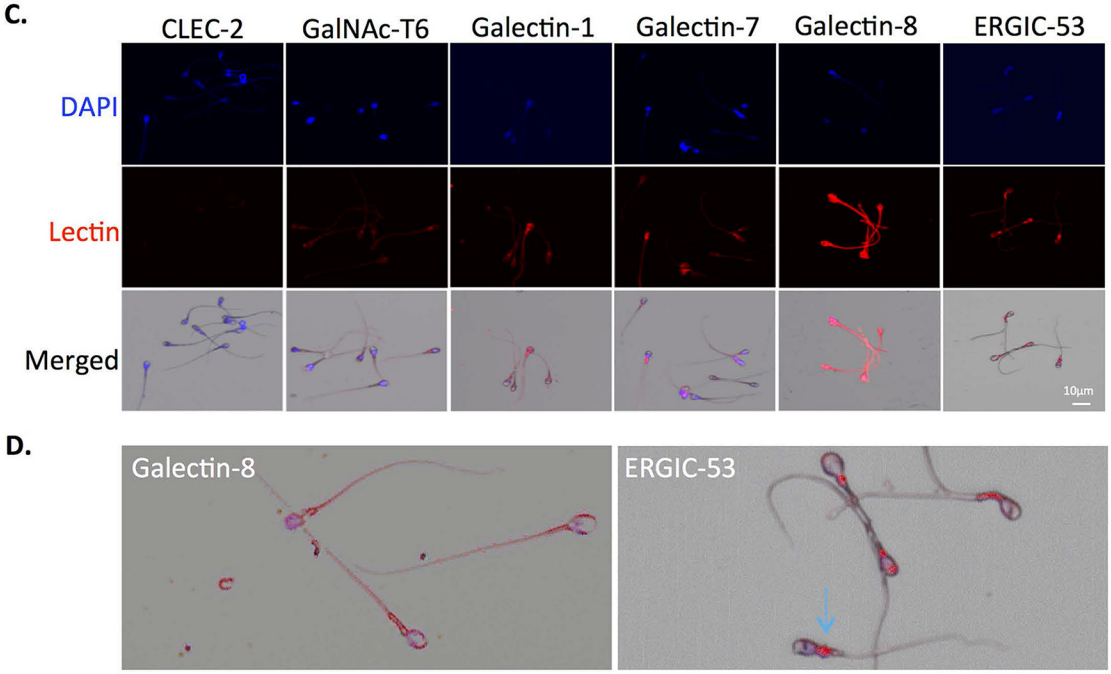


FIG. 4. **Human sperms show significant binding with several of the human lectins.** *A*, The lectin and lectin-like proteins on the microarray bound the human sperms. The sperms were fluorescent labeled and incubated on the microarray. After removing of the free sperms, the results were recorded with a microarray scanner. *B*, Flow cytometry analysis confirmed the positive binding to sperms with galectin-1, galectin-7, galectin-8, GalNAc-T6 and ERGIC 53. A lectin didn't show positive binding with sperm on the microarray, *i.e.* CLEC-2, was included as a negative control. *C*, Incubation of biotinylated galectin-1, galectin-7, galectin-8, GalNAc-T6 and ERGIC 53, followed by the addition of Cy3-conjugated streptavidin and direct inspection with microscopy further confirmed the sperm-lectin bind. CLEC-2 was included as a negative control. *D*, The sperm glycans or glycoproteins that galectin-8 and ERGIC 53 bound to showed different distribution on the sperm surface.

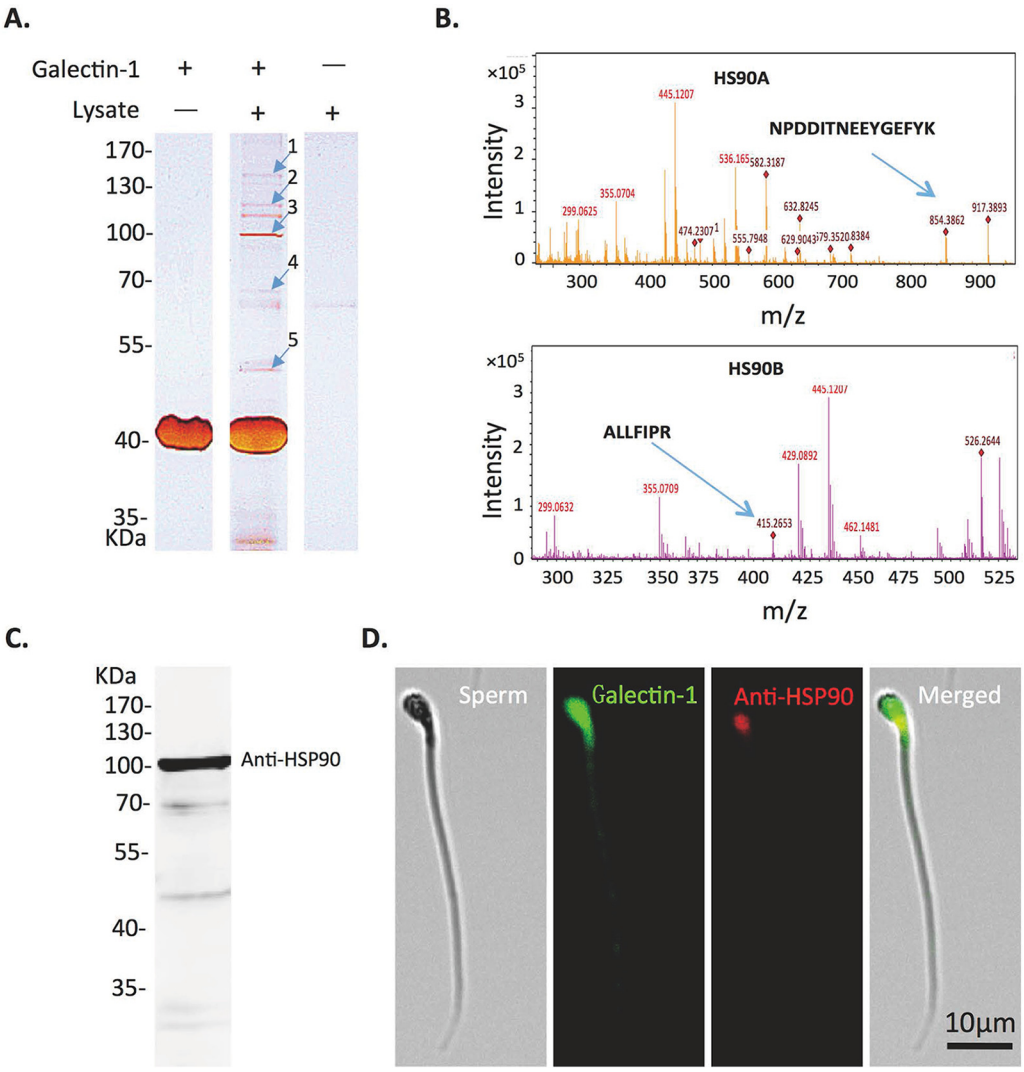


FIG. 5. **The identification of Galectin-1 recognized membrane-associated proteins by LC-MS/MS analysis.** *A*, LC-MS/MS was used to identify the galactosylations of the membrane-associated proteins captured by galectin-1. *B*, LC-MS/MS spectrum of two peptides. The corresponding proteins were identified as HSP90A and HSP90B. *C*, Anti-HSP90 antibody specific recognized HSP90 from sperm cell lysate. *D*, Localization of HSP90 with galectin-1 and anti-HSP90 antibody. Sperms were incubated with biotinylated galectin-1 and anti-HSP90 antibody simultaneously, the results of galcetin-1 and HSP90 were then visualized by Cy3-streptavidin and a Cy5 conjugated second antibody specific for the anti-HSP90, respectively.

TABLE II
Identification of proteins captured by galectin-1 with mass spectrometry

NO.	Accession	Protein	MW [kDa]	Scores	Peptides	Location	Glycosylation
1	MUC6	Mucin6	256.9	193	8	Secreted (47)	O-glycosylation (48)
2	ACE	Angiotensin-converting enzyme	149.6	102.0	9	Membrane (49, 50)	N-glycosylation (51)
3	HS90AA1	Heat shock protein HSP 90-alpha	84.6	496.1	19	Membrane (52)	Unknown
3	HS90AB1	Heat shock protein HSP 90-beta	83.2	330.6	15	Secreted (53)	O-glycosylation (54, 55)
4	HSPA1A	Heat shock-related 70 kDa protein 2	70.0	530.9	15	Membrane (52)	Glycosylation (56)
4	HSPA1L	Heat shock 70 kDa protein 1-like	70.3	178.5	9	Membrane (52)	Unknown
4	HSPA1A/1B	Heat shock 70 kDa protein 1A/1B	70.0	120.9	6	Secreted (57)	Glycosylation (56)
5	TUBB4B	Tubulin beta-4B chain	49.8	182.7	15	Surface (52)	Unknown

trophoblast cells during placentation (34, 35). Our data show that galectin-1 primarily binds to heat shock proteins in the sperm neck and other glycoproteins of sperm head. This indicates that HSP90 is glycosylated in sperm and may plays critical roles in maintain sperm activity. Indeed, previous studies found that the HSP90 protein level is positively correlated

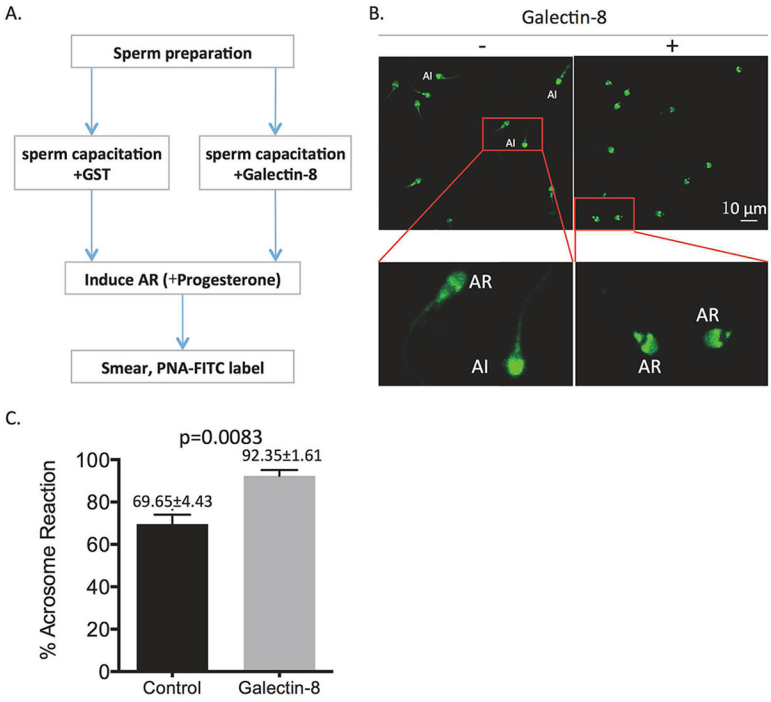


FIG. 6. **Galectin-8 promotes acrosomal reaction through enhancing of sperm capacitation.** *A*, The workflow of the assay for monitoring acrosomal reaction upon the treatment with galectin-8. Briefly, human sperms were exposed to galectin-8 during capacitation. Sperms were also treated with GST only as control. The sperms were then incubated with progesterone to induce acrosomal reaction. The sperms were then stained with FITC conjugated PNA to monitor the acrosomal reaction. *B*, PNA stained acrosomes from galectin-8 treated sperms and that of the GST treated control. *C*, Quantitation of the acrosomal reaction. Results represent means ± S.D. of 3 independent experiments.

with sperm quality (36). In other earlier work, Gustavo Vasen *et al.*, (37) found that galectin-1 is expressed in the epididymis and associates with sperm during epididymal maturation. Exposure of sperm to galectin-1 resulted in glycan-dependent modulation of AR. Using galectin-1 deficient mice, they showed that galectin-1 is essential for sperm to gain the capability of fertilization (37). However, the underlying mechanism was not clear. Based on our results, one plausible explanation is that galectin-1 affects fertilization through interaction with glycoprotein HSP90 on sperm neck.

Galectin-8 is a tandem repeat galectin with two carbohydrate recognition domains (CRDs). Each CRD of Gal-8 shows distinct glycan specificity: the N-terminal domain of galectin-8 recognizing sulfated and sialylated glycans and the C-terminal domain of galectin-8 recognizes blood group antigens and polyLacNAc glycans (38). Galectin-8 is expressed in female and male reproductive systems (35). The protein level of galectin-8 is low during the proliferative phase, and high during the lutheal phase of the female endometrium (39). Galectins are abundantly expressed in the female reproductive tract, and they play roles during implantation and placentation (40). Thus it is possible that galectin-8 expressed on the inner surface of genital tract may interact with the glycans on the sperms, promote sperm capacitation and then the acrosome reaction or promote acrosome reaction directly.

Galectin-7 is a "proto-typical" galectin, like galectin-1, containing a single CRD of ~130 amino acids (41). It can be found in female reproductive system (35) and its expression changes in some cancers of the reproductive tract, including that of the ovary and uterus. Endometrial epithelium can produce galectin-7 and women with a history of miscarriage have abnormally elevated levels of galectin-7 in the endometrium (42). GalNAc-T6 is one of the members of the UDP-N-acetyl-alpha-D-galactosamine: polypeptide N-acetylgalactosaminyl-transferase family of enzymes. GalNAc-Ts catalyze the transfer of GalNAc to serine and threonine residues on target proteins to initiate mucin-type O-linked glycosylation in the Golgi apparatus. It is known that GalNAc-T6 expresses in spermatozoa but not in acrosome (43). ERGIC-53 is a mannose-specific membrane lectin operating as a cargo receptor for the transport of glycoproteins from the ER to the endoplasmic reticulum-golgi intermediate compartment (ERGIC) (44). Functional inactivation of ERGIC-53 in *Caenorhabditis elegans* leads to an egg maturation defect and reduced fertility.

The major advantage of our human lectin microarray over the traditional plant lectin microarray lies in the human lectins themselves. The lectins and lectin-like proteins all naturally exist in the human body. Hence, it is quite likely that these proteins exhibit a greater selectivity and specificity in recognizing complex glycan structures in our body than the plant lectins, making them more suitable for analysis of human samples or human pathogens. Nonetheless, the current version of this human lectin microarray also possesses several disadvantages. First, there are less than 50% of the known human lectins and lectin-like proteins on the microarray. When we first initiated this study, there were about 100 known human lectins and lectin-like proteins. However, because of the lack of available cDNA clones and the difficulties that we encountered during protein expression and purification, only 60 proteins were included in the current version. We will include a more comprehensive set of human lectins and lec-

tin-like proteins in the next version by adding newly identified ones as well as trying alternative expression and purification strategies. Second, it is difficult to maintain the activity of the membrane localizing human lectins and lectin-like proteins. Over 60% of the presently known human lectins are membrane proteins. It is well known that even successfully purification, the activity of these proteins is usually hard to retain and the proteins are not stable for long-term storage. Though we have demonstrated that many of the proteins on the microarray are active, it was not surprising that some of the proteins were inactive or retained very low activities. Additionally, after the microarrays were fabricated, they could be stored under −80 °C at most for 6 months without significant performance lost (data not shown). The key for solving this problem is to keep the natural confirmation of the membrane localized lectins. One possible solution is to stabilize the membrane proteins with nanodiscs during purification and immobilization (45). Alternatively, the so called Virion Display (VirD) technology may also be a viable option, in which the membrane proteins are expressed in mammalian cells and presented on the membrane of purified virions (46).

Taken together, we have constructed a human lectin microarray, which, to our knowledge, is the first of its kind. We have successfully applied the microarray in the analysis of human sperms and identified five proteins that could specifically bind sperms. These results suggest that interactions of lectins and sperms may play critical roles in sperm maturation and fertilization. Our microarray is easily expanded with either more human lectins or with plant lectins. As a powerful tool, we envision that our microarray will be widely applied in a variety of human glycosylation related studies.

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Authors declare that we have no conflict of interest.

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